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14. ABSTRACT

Cancer cells have to break through basement membrane (BM) to metastasize. Laminin-5 is one of the major components of BM. It is composed of three chains α3, β3 and γ2. The DIII domain, which could be released from Ln-5 γ2 chain by MMPs processing, can bind to EGFR and activates the downstream signaling pathway. The role of Ln-5 in cancer progression is still unclear. There are paradoxical data in regard of the role of Ln-5 in cancer progression. Both increased and decreased expression levels of Ln-5 subchains are reported in the literature. The fact that γ2 chain exists in two different forms (as a secreted monomer, or as a part of the Ln-5 heterotrimer) leads us to hypothesize that those two forms may play different roles in cancer progression. In this proposal we will determine whether Ln-5 γ2 monomer is positively correlated with breast cancer tumorigenecity. We will also determine the role of Ln-5 γ2 chain in cancer progression when it is in the context of Ln-5 heterotrimer.

15. SUBJECT TERMS
Laminin-5(Ln-5), breast cancer, Ln-5 gamma2 monomer, Ln-5 heterotrimer, cancer progression

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Introduction

To metastasize, cancer cells have to break through the basement membrane. Ln-5 is one of the basement membrane proteins, consisting of three chains α3, β3 and γ2. Ln-5 γ2 chain contains DIII domain, a functional EGFR ligand, which can be released by MMP processing. It has been suggested by our lab that DIII domain may facilitate cancer progression by preventing anoikis. What we noticed is that there are paradoxical data in regard of the role of Ln-5 in cancer progression. For example, both the increased and decreased expression levels of Ln-5 subchains are reported in the literature.

The fact that γ2 chain exists in two different forms (as a secreted monomer, or as a part of the Ln-5 heterotrimer) leads us to hypothesize that those two forms may play different roles in cancer progression.

Therefore, the original aims are expanded and modified as the following two: A). To determine if the expression of Ln-5 γ2 monomer is correlated with breast cancer cell line tumorigenecity. B). To determine the role of Ln-5 γ2 chain in cancer progression when it is in the context of Ln-5 heterotrimer. To address those two aims, we carried our study using breast cancer cell lines MCF10A and CA1a together with bladder carcinoma cell line 804G.

Body

A) To determine if the expression of Ln-5 γ2 monomer is correlated with breast cancer cell line tumorigenecity. (This work is done in collaboration with Cherise Guess， Kam Yoonseok and Mohammed Hassanein)

It was reported in previous progress report that CA1a is very likely to secret γ2 monomers since it has low β3/γ2 ratio of the CA1a cells is lower than the β3/γ2 ratio of the MCF10A cells, which suggest. The status of γ2 monomer in those two cell lines will be further confirmed by western blotting using γ2 monomer specific antibody 1H3[1]. To decrease Ln-5 γ2 monomer in CA1a, Ln-5 γ2 chains will be knocked down using shRNA construct (Open biosystem), which has been tested in MCF10A.

Ln-5 γ2 monomer has been implicated in supporting cell survival and invasion. To better design our assays to characterize our knockdown cells, we checked the basal activities of parental CA1a in cell survival, migration and invasion assays. Cell survival was checked by counting dead/live cell number over four days in the presence of 10% or 0% serum. Cells were trypsinized from the plate every 12hours. Live and dead Cell numbers were counted on hemacytometer after trypan blue staining. What we found is that CA1a cells keep proliferating in the absence of serum, and the viability of CA1a persists to be similar under 10% and 0% serum conditions.(Fig.1)

which is compared to the original wound size. The higher after/ before ratio of island sizes, the more migration the cells do.

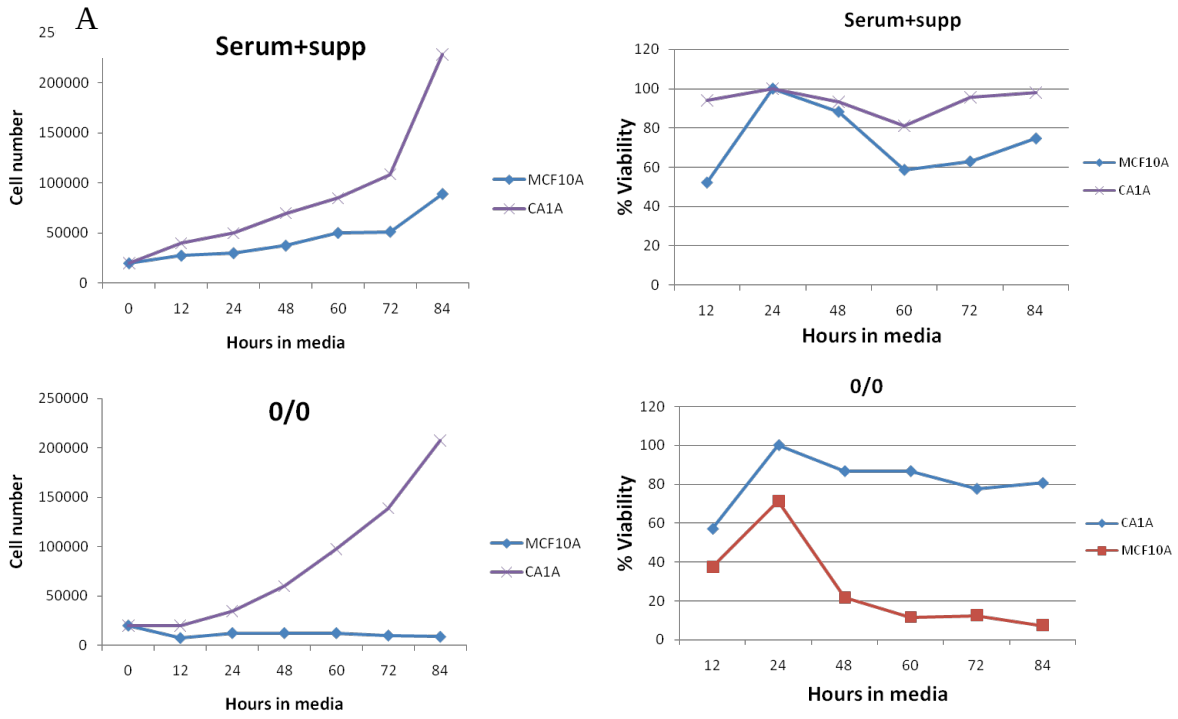
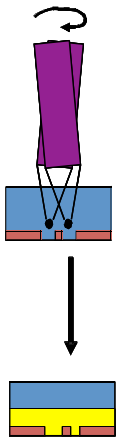


Fig.1 A.Cell growth curve with 10% serum. B. cell growth curve with 0% serum C. Viability of cells with 10% serum D. Viability of cells with 0% serum

Cell migration abilities in 10% or 0% serum were determined in modified circular wound healing assay (CWA). (Kam Y et. al. manuscript submitted) In this modified CWA, wounds were made as shown in Fig. 2. Briefly, instead of making a circular wound, an island of cells is left in the middle of the wound. The size of "island" was measured after allowing cells to migrate 12 hrs,



Cell invasion abilities were determined by overlaying 50% Matrigel to the circular wound, which denoted as circular would invasion assay. (CIA) Quantification of CIA is the same as CWA. As shown in Fig. 3, the migration and invasion activity of CA1a is higher than the non-tumorigenic cell line MCF10A.

Fig. 2 Brown: cell monolayer Yellow: culture medium

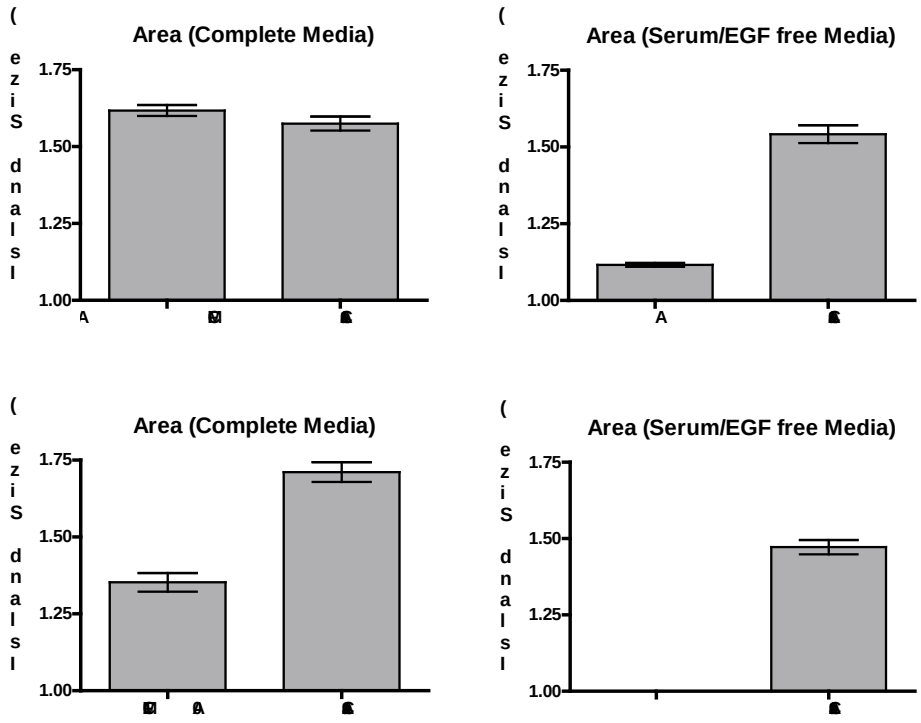


Fig. 3 Cell migration and invasion abilities of MCF10A and CA1a in CWA assay.
Top: CWA, Bottom: CIA; Left: 0% serum. Right: 10% serum

Our preliminary data on cell survival, migration and invasion assays suggest that CA1a is an aggressive cell line compared to MCF10A. CA1a is not sensitive to serum and EGF deprivation which is a "tough" condition for non-tumorigenic cell line MCF10A. We suspect that γ2 monomers secreted by CA1a which can activate EGFR signaling pathway, play a role to help cells to adapt to the "tough" condition. CA1a-ctrl and CA1a-kd will be generated and examined in those assays soon. The tumorigenicity of CA1a-ctrl and kd will be determined in kidney capsule model as described in previous report.

It has been reported by our lab that DIII domain in Ln-5 γ2 chain can activate EGFR signaling pathway. To determine whether DIII domain is responsible for the possible phenotypes induced by removing Ln-5 γ2 monomer, a mutant human γ2 containing no DIII domain was constructed.

B) To determine the role of Ln-5 γ2 chain in cancer progression in the context of Ln-5 heterotrimer.

Last year, we reported that removing Ln-5 γ2 in bladder carcinoma cell line 804G, which synthesize Ln-5 heterotrimers, induced increased tumor growth significantly. The phenotypes induced by removing γ2 in 804G are summarized in table 1.The highlighted areas are new data since last progress report, which are described below.

Metabolic rates: The more tumorigenic the cells are, the higher metabolic rates the cells have. We compared the metabolic rated between 804G-ctrl and 804G-kd in collaboration with Cliffel's lab (Vanderbilt University). Four parameters were examined: medium acidifying rate (ECAL), glucose uptake rate (GU), lactate produce rate (LP) and O_2 uptake rate (OU). (Fig.3 A)[2] Consistent with our in vivo data, 804G-kd cells have much higher rate than 804G-ctrl for all of those four parameters. Interestingly, the glucose uptake rate in 804G-kd was decreased by exogenous Ln-5 (1ug/ml) coating to the similar level as 804G-ctrl. (Fig.4B)

Traits	Ctrl	Kd γ 2
Proliferation rate	Higher	Lower
E-cad localization	Cell-cell junction	Cytoplasmic
EMT	No	Yes
Metabolic rates	Lower	Higher
Cell-cell adhesion strength	Lower	Higher
Migration velocity	Higher	Lower
Collagen gel migration	Collective	Single cell
ECM degradation (invadopodia)	Lower	Higher
Tumor	Small	Large

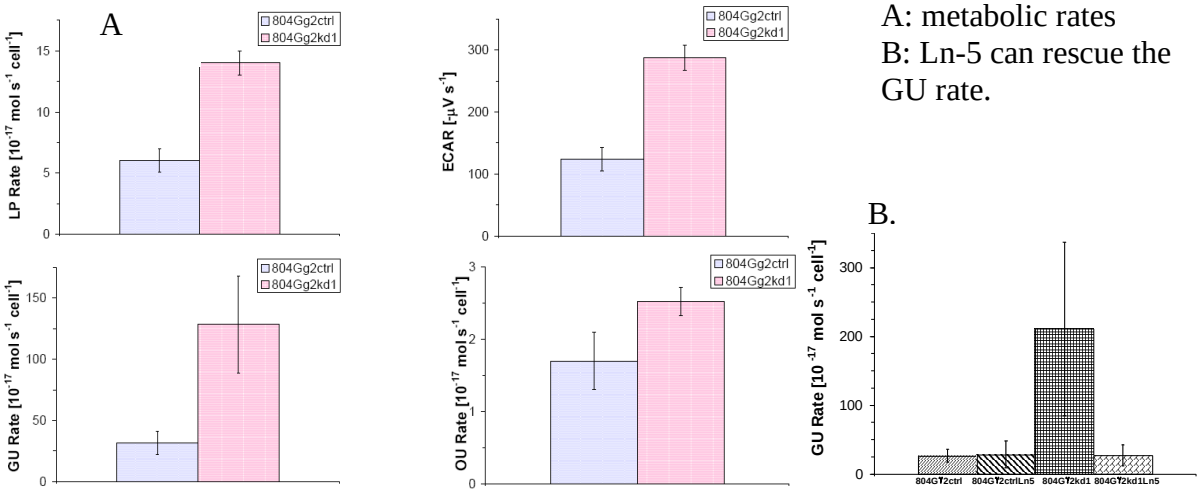


Fig. 4
A: metabolic rates
B: Ln-5 can rescue the GU rate.

Cell-cell adhesion: The physical strength of cell-cell adhesion was measured using two different assays, hangingdrop assay and dispase assay. In hanging drop assay, 15000 cells were plated in 30ul of culture medium in hanging drop and incubated overnight at 37℃. The cell aggregations in the hanging drop were then disrupted by mechanical strength through pipette. The single cell numbers were compared between before and after applying physical strength. The more single cells after applying mechanical strength, the less strong the cell-cell adhesions are. Instead of culturing cells in the hanging drop, dispase assay allows cells to be cultured in attachment to the culture plate. Disapse is an enzyme which cleaves the cell membrane proteins to break cell-matrix adhesion. Thus, monolayer of cells could be lifted from tissue culture plate by dispase treatment. Mechanical strength is applied to the monolayer in suspension to disrupt cell-cell

adhesion, which will result in single cells. Dispase assay protocol is modified from Calautti et al, JCB, 1998. [3] Despite less E-cads at the cell-cell junctions, 804G-kd cells have higher cell-cell adhesion strength in both assays.

Collagen gel migration assay In this assay, instead of grafting the collagen gel (see previous progress report for details) under the kidney capsule, the collagen gels were left in the tissue culture plates in the incubator for 4 days. 804G-kd and 804G-ctrl were different in the migration pattern.As shown in Fig.5, 804G-ctrl showed collective migration pattern while 804G-kd showed single-cell migration pattern.

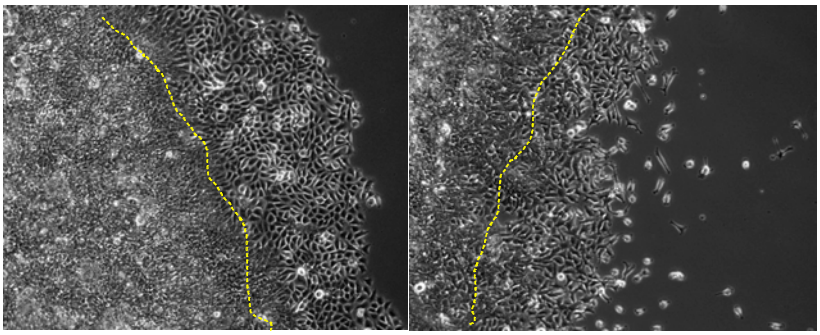


Fig. 5 804G-ctrl and 804G-kd have different migration pattern in collagen gel assay.
Left: 804G-ctrl
Right: 804G-kd
Dotted lines delinate the borders of the collagen gel.

For task B, we also proposed to knock down Ln-5 γ2 chain in MCF10A cells. MCF10A-ctrl and MCF10A-kd cells were generated using the shRNA construct mentioned in part A. As shown in the fluorescence immunostaining, in the MCF10A-kd cell population, most of cells are Ln-5 γ2 negative (stained by anti-Ln-5 γ2 polyclonal antibody 2778). (Fig.6)

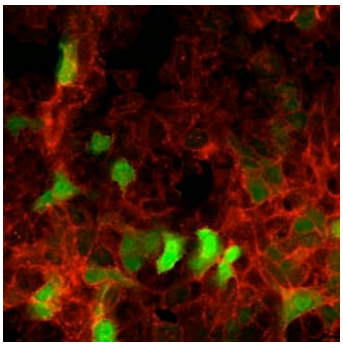


Fig.6
Ln-5 γ2 was detected by 2778. Green: 2778,
Red: Phalloidin (F-actin).

The cell invasion, migration and cell survival of the parental cell line MCF10A were determined in the way as mentioned in part A. Apparently; MCF10A cells were not as aggressive as CA-1a in those assays. As shown in Fig. 1-3, MCF10A cells were sensitive to serum deprivation. In the absence of serum, less than 10% MCF10A cells survived after four days culture. The invasion and migration activities were also dramatically decreased in the absence of serum. MCF10A-ctrl and MCF10A-kd will be compared in those two assays. Based on our study in 804G cells, we expect that MCF10A-kd will be more aggressive than ctrl in those assays.

Preliminary mice experiment showed that neither MCF10A-kd nor MCF10A-ctrl had significant tumor growth or local invasion to the kidney in one month. We will wait longer to see if MCF10A-ctrl and/or kd cells could be tumorigenic. However, MCF10A is known as a non-

tumorigenic cell line. There is possibility that removing Ln-5 γ2 is not enough to turn MCF10A into a highly tumorigenic or even tumorigenic cell line.

Key Research Accomplishments

RT-PCR showed that MCF10ACA1a (tumorigenic) has more γ2 monomer than MCF10A (non tumorigenic).

Cell migration, invasion and survival of parental cell line CA1a and MCF10A were determined in two conditions (10% and 0% serum). Our data suggest that CA1a is not sensitive to serum deprivation, while MCF10A is. It suggests that serum concentration might be critical for experimental design to compare γ2-ctrl and γ2-kd cells.

Human Ln-5 γ2 expression vector which has DIII domain deletion was constructed.

Downregulation of Ln-5 γ2 by shRNA in 804G cells induces significant higher tumorigenicity. Consistently, 804G-kd cells acquire higher metabolic rates than 804G-ctrl cells. Interestingly, exogenous Ln-5 coating rescued the glucose uptake rate.

Two cell lines were made, MCF10A-ctrl and MCF10A-kd.

Reportable Outcomes

The work in this project was presented in the department seminars at Vanderbilt University in 2006.

The poster was presented in the American Society for Matrix Biology Biennial National Meeting 2006. (Mentioned in last progress report)

The work in this project has been presented in the department seminars at Vanderblit University in 2007.

One manuscript from this project is in preparation.

Conclusion

It has been suggested in the literature that overexpression of Ln-5 γ2 chain had been associated with increased tumorigenesis, in breast as well as other cancers. However, γ2 chain deletion in 804G cell, dramatically upregulates its tumorigenicity in *in vivo* models. The distinction between those two cases is the status of γ2 chain, monomer or in the context of Ln-5 heterotrimer.

We hypothesize that Ln-5 heterotrimers have a tumor suppressor role, γ2 chain monomers instead have a tumor promoting role.

Future direction

CA1a ctrl and kd cells will be made. And they will be compared in the migration, invasion and cell survival assays in 10% and 0% serum. The optimal time for CA1a to develop tumor in mice will be determined.

For part B, MCF10A kd and ctrl cells will be compared in the migration, invasion and cell survival assays in 10% and 0% serum as mentioned in part A. We will put MCF10Actrl and kd into the kidney capsule model as well.

For 804G cell system, we will try to understand the mechanism by removing Ln-5 receptors.

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